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Development of electrochemical inhibition biosensor based on bacteria for detection of environmental pollutants☆

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ABSTRACT

The main aim of this work is to develop a simple inhibition electrochemical sensor array for detection of heavy metals using bacteria. A series of electrical measurements (cyclic voltammograms) were carried out on samples of two types of bacteria, namely *Escherichia coli* and *Shewanella oneidensis* along with optical measurements (fluorescence microscopy, optical density, and flow cytometry) for comparison purposes. As a first step, a correlation between DC electrical conductivity and bacteria concentration in solution was established. The study of the effect of heavy metal ions (Hg^{2+}) on DC electrical characteristics of bacteria revealed a possibility of pattern recognition of inhibition agents. Electrical properties of bacteria in solution were compared to those for immobilized bacteria.

1. Introduction

Heavy metal pollution is a problem associated with areas of intensive industry. Zinc, copper, and lead are three of the most common heavy metals released from road travel. Lead concentrations, however, have been decreasing consistently since leaded gasoline was discontinued [1]. The existing high-tech methods of their detection are usually expensive and laboratory based. This work is a part of ongoing research targeting the development of novel, simple, and cost effective methods for monitoring environmental pollutants, particularly pesticides, petrochemicals and heavy metals being common contaminants of water resources. It is known that microorganisms are very sensitive to heavy metals [2,3]. The use of microorganisms for assessment of general toxicity of aqueous environment was reported previously [4]. Identification of the types of pollutants in the environment and the evaluation of their concentration is much more difficult task which is impossible to solve using a single inhibition type of sensor. However, the sensor array approaches utilising several types of bacteria being inhibited differently by different types of pollutants could solve the above problem. Electrochemical measurements were successfully used for studying electrical properties of cells deposited on screen printed gold electrodes and showed great prospects of using such cell-based sensors for detection of various pollutants [5,6]. Our previous experiments went further and expanded this idea to more complex bio-objects such as bacteria. Using two types of bacteria, *Escherichia coli* and *Deinococcus radiodurans*, we confirmed the principles of inhibition sensor array which was capable of differentiation between radionuclide and heavy metal pollutants [2,7]. In this work, we used simple electrochemical measurements for establishing the correlation between conductivity of liquid bacteria samples and immobilized bacteria, and studying the effect of heavy metal ions (Hg^{2+}) on them. In addition to *E. coli* bacteria, we used another type of bacteria, *Shewanella oneidensis* known by its high resistance to heavy metals. The main focus of this work is on electrical characterisation of both free bacteria in solution and bacteria immobilized on the surface of metal electrodes.

2. Experimental methodology

2.1. Bacteria sample preparation

Two types of bacteria were selected for this work: *Escherichia coli* (*E. coli*), which is quite sensitive to different environmental pollutants, and *Shewanella oneidensis* (*S. oneidensis*) known by its extreme resistance to heavy metals. LB (Luria-Bertain) broth was used as a medium [8] for both bacterial cell cultures. Both types of bacteria and respective growth media were acquired from Sigma-Aldrich Co. Other chemicals, i.e. $HgCl_2$ salt and poly L-lysine (PLl) were also purchased from Sigma-Aldrich Co. Cultivation of bacteria was performed in several stages.

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The first step was to cultivate a specific strain of bacteria in Petri dish containing solid broth agar, in order to use it as a bacteria source in future. In the second stage, one colony of bacteria was added into a sterile flask containing 50 ml of liquid broth. Finally, the flask containing the bacterial culture was placed inside shaking incubator operating at 150 rpm shaking speed. The incubation temperatures were 30 °C for *S. oneidensis* and 37 °C for *E. coli*. Bacteria start growing after 16 h for *E. coli* and 24 h for *S. oneidensis*.

2.2. Experimental procedures

To study the inhibition effect of heavy metals on the above bacteria, $HgCl_2$ salt (from Sigma-Aldrich) was selected. Solutions in different concentrations (0.1, 1, 10, 100 mM) of $HgCl_2$ were prepared by multiple dilution of 1 M stock solution of $HgCl_2$ in deionised water. Bacteria samples were mixed with $HgCl_2$ solutions in 1:1 ratio and kept incubated for 2 h.

The effect of heavy metal on the bacterial density was examined and analyzed using three different optical experimental techniques: fluorescence microscopy, UV–visible spectrophotometry, and flow cytometry. GALLIOS flow cytometer BECKMAN-COULTERPN A75199AA instrument was used for counting the percentage of live and dead bacteria after colouring bacteria samples with L7012 Live/Dead Bacterial Viability Kit. Fluorescence microscopy measurements were performed using Olympus-BX60 instrument. In this study, bacterial samples were also stained using L7012 Live/Dead (L/D) BacLight Bacterial Viability Kit [9], which is a mixture of (SYTO-9) green fluorescence nucleic acid stain and the red fluorescence nucleic acid stain propidium iodide. The cultivated bacteria density and changes in the live bacteria counts after exposure to pollution were monitored using optical density photometer (6715 UV/Vis Spectrophotometer JENWAY OD600). The electrochemical measurements, i.e. cyclic voltammograms (CV), were carried using gold screen printed three-electrode assemblies and DropSens microSTAT200 potentiostat. The same CV measurements were carried on both types of bacteria immobilized on the surface of gold electrodes via poly L-lysine (PLl) [10]. For that purpose, the surface of gold was modified in a 1:1000 mixture of PLl (1 mg/ml) and deionised water for 1 h at 37 °C. Then bacteria were immobilized by dropping stock solutions of either *E. coli* or *S. oneidensis* in LB broth on the modified electrodes, keeping it there for 1 h, then washing out non-bound bacteria with phosphate buffer solution (PBS). The CV measurements of the electrodes with freshly immobilized bacteria were carried out in PBS both before and after treatment with $HgCl_2$ in different concentrations.

3. Experimental results and discussion

3.1. Optical measurements

The numbers of live and dead bacteria were determined with fluorescence microscopy and OD600 similarly to that described in [7]. Live and dead bacteria appeared as green and red dots, respectively, on fluorescence microscopy images in Figs. 1 and 2 for *E. coli* and *S. oneidensis*, respectively.

It is clear, that the exposure to $HgCl_2$ reduces the number of live (green) bacteria and increases the dead ones (red), while *S. oneidensis* bacteria are much less affected.

Similar and even more pronounced pattern was observed in flow cytometry experiments where bacteria were stained with the same L7012 Live/Dead Bacterial Viability Kit and appeared on the graphs in Fig. 3 as blue (live) and orange (dead) dots. The increase in the dead *E. coli* bacteria count after exposure to $HgCl_2$ salt is visually apparent. Image analysis of Fig. 3A(b) yields the percentage of live *E. coli* 43.88% and 56.12% for dead ones.

In addition to that, dead *E. coli* bacteria appear mostly in bottom-left quadrant of the graph in Fig. 3A(b) indicating the increase in the bacteria size most-likely due to rapture of cell membranes. Contrary, *S. oneidensis* bacteria were affected much less, the percentages of live and dead bacteria after exposure to 1 M $HgCl_2$ solution were 83.36% and 16.64%, respectively. Again, dead bacteria appeared slightly enlarged since they were shifted to the bottom-left in Fig. 3B(b).

The result of optical density measurements of both bacteria samples and the effect of their treatment with $HgCl_2$ salt of different concentrations are shown in Table 1. The bacteria density was assessed (and presented as absorbance) by losses of light intensity in the middle of visible range (600 nm) as a result of light scattering on bacteria. The reduction in optical density upon increasing the $HgCl_2$ concentration is much more pronounced for *E. coli* than that for *S. oneidensis*.

Among the three optical methods used, flow cytometry appeared to be the most reliable and not affected by different motility of *E. coli* and *S. oneidensis*. It is known that dead *E. coli* bacteria are not motile and tend to sediment which may affect the results of static fluorescent microscopy and optical density measurements. Nevertheless, the results of optical testing of bacteria samples provided a background for further study using much simpler electrochemical method.

3.2. Electrochemical measurements on liquid bacteria samples

The electrochemical measurements of bacterial samples were carried out using DropSens potentiostat and screen printed gold electrodes. Potential was recorded against Ag/AgCl reference electrode. Typical cyclic voltammograms for *E. coli* and *S. oneidensis* of different concentrations (i.e. dilutions with LB broth) are shown in Fig. 4.

Generally, the CV graphs in Fig. 4 are almost featureless in the selected voltage range from −0.5 V to +0.5 V which was chosen deliberately in order to avoid electrochemical reactions on the electrodes. Slight increase in the cathode current at −0.2 V indicates the beginning of hydrogen reduction.

The values of cathode current at −0.5 V appear to decrease with the increase in bacteria concentration (or dilution ratio 1:10, 1:5, 1:2, 1:1) with the largest current shown for clear LB broth and the lowest for

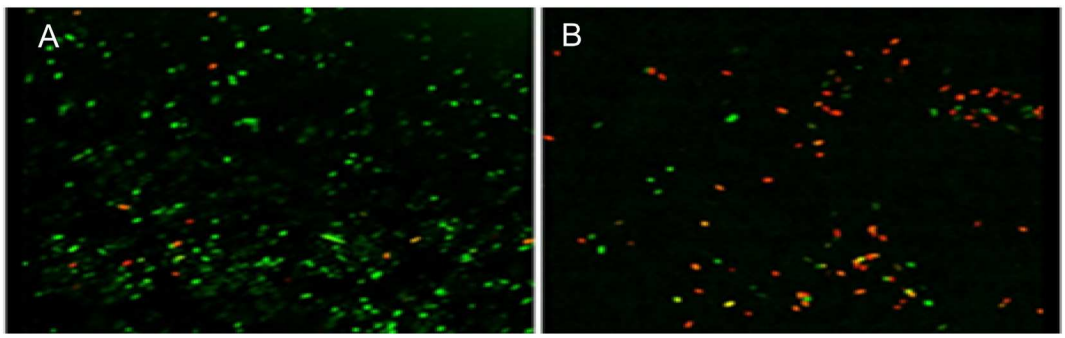

Fig. 1. Fluorescence microscopy images of *E. coli* before (A) and after (B) treatment with $HgCl_2$ salt (1 M).

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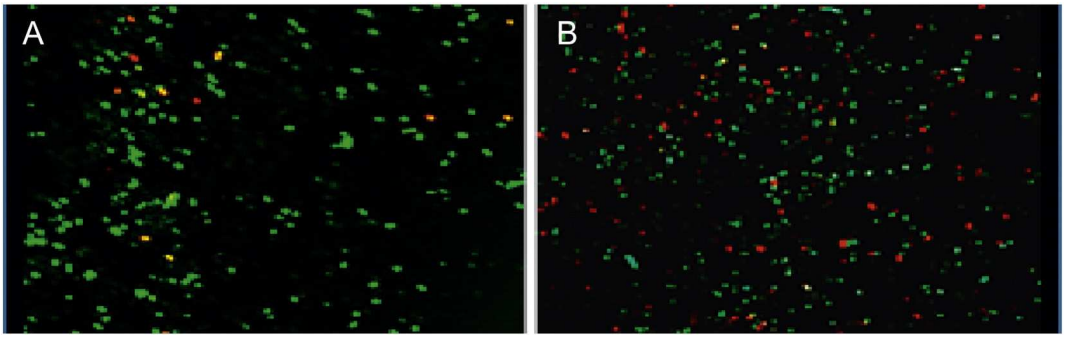

Fig. 2. Fluorescence microscopy images of *S. oneidensis* before (A) and after (B) treatment with $HgCl_2$ salt (1 M).

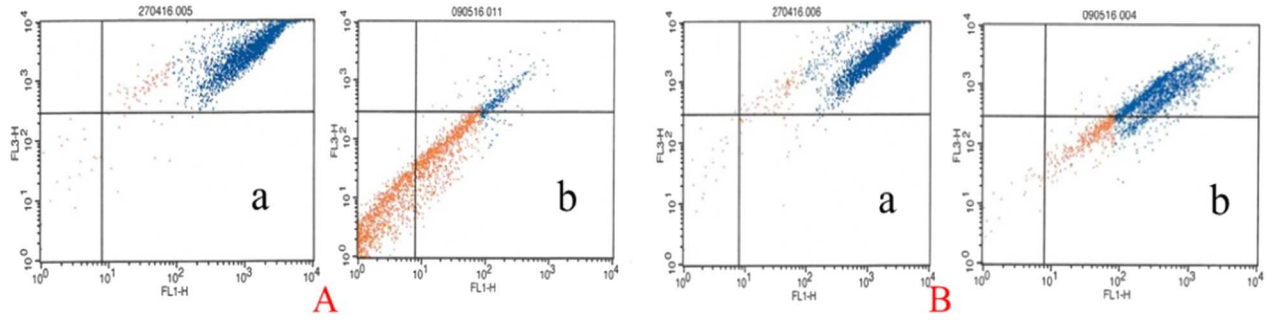

Fig. 3. Flow cytometry results for *E. coli* bacteria (A) and *S. oneidensis* (B); graphs (a) and (b) were obtained, respectively, before and after treatment with $HgCl_2$ (1 M).

bacteria stock solution. That means that bacteria adsorbed on the surface of gold electrodes act as insulating layer reducing the current. These results are very important since they establish a correlation between the values of cathode current and bacteria concentration in solution.

The next step was to study the effect of Hg^{2+} ions on CV characteristics of both *S. oneidensis* and *E. coli* bacteria. A series of CV measurements were carried out on liquid samples of bacteria stock solution in LB broth which was mixed in 1:1 ration with different concentrations of $HgCl_2$ and kept for 2 h prior to CV measurements. The results of these measurements in Fig. 5 show substantial increase in cathode current (I_c) for *E. coli* samples (Fig. 5A) upon increasing the concentration of $HgCl_2$ while this effect is much less pronounced for *S. oneidensis* samples (Fig. 5B). In addition to that, the oxidation and reduction peaks appeared on CV curves for *S. oneidensis* samples in Fig. 5A which are definitely related to electrochemical reactions associated with the presence of $HgCl_2$. The anodic peak increases with the increase in $HgCl_2$ concentration. However, such electrochemical reactions do not appear on *E. coli* samples in Fig. 5B. It is known that heavy metal salts act as co-factor for *S. oneidensis* bacteria growth [11], so the oxidation peak at about 0.2 V on Fig. 5A is most-likely related to the interaction of $HgCl_2$ with *S. oneidensis*.

When analysing the effect of heavy metal salts on CV characteristics of liquid bacteria samples the effect of extra Hg^{2+} and Cl^- ions on conductivity of liquid medium has to be taken into account. In order to find out the true effect of heavy metal ions on bacteria, the values of cathode current (I_c) of bacteria samples has to be normalized by the reference current I_{ref} of LB broth diluted 1:1 with particular concentration of $HgCl_2$. The values of relative changes of cathode current $\Delta I_c/I_c = (I_c - I_{ref})/I_{ref}$ at −0.5 V of both *S. oneidensis* and *E. coli* bacteria are plotted in Fig. 6 against the concentration of $HgCl_2$.

As one can see the effects of $HgCl_2$ on *S. oneidensis* and *E. coli* are completely different: $\Delta I_c/I_{ref}$ goes up with the increase in $HgCl_2$ concentration for *E. coli* which means that *E. coli* bacteria inhibited by Hg^{2+} ions becoming less electrically resisting, while $\Delta I_c/I_{ref}$ is almost flat at low concentrations of $HgCl_2$ and slightly increases at high concentration of 1 M. This means that *S. oneidensis* are practically not affected by $HgCl_2$ low concentrations but inhibited at high concentrations. This is a very promising result showing a possibility of pattern recognition of heavy metals using the two bacteria.

Table 1
The results of OD600 for both bacteria samples which exposure to $HgCl_2$ for 2 h.

	$HgCl_2$ concentration					
Bacteria	Before treatment	0.1 mM	1 mM	10 mM	100 mM	1 M
E. coli	0.813	0.773	0.712	0.637	0.416	0.356
S. oneidensis	0.827	0.869	0.861	0.793	0.832	0.753

3.3. Electrochemical measurements on immobilized bacteria

The results given in the previous section are important as a further step towards the development of bacteria-based inhibition sensor array, but it is still far away from real sensor development. Dealing with liquid bacteria samples is not the way forward because of natural variations of bacteria concentration even in laboratory samples not to mention “real” samples taken for analysis. The problem of having a reliable reference for such measurements is a very difficult one. It would be much more useful for real sensor development to use bacteria immobilized on the electrode surface.

In this work, both selected bacteria, e.g. *S. oneidensis* and *E. coli*, were immobilized on the surface of gold electrodes via poly L-lysine as described in more detail in Section 2.2. Fig. 7 shows a series of CV measurements carried out in PBS on gold electrodes with *S. oneidensis* and *E. coli* immobilized from their respective solutions of different concentrations in LB broth (stock solution and 1:2, 1:5, 1:10 dilutions with LB broth were used). Both graphs show characteristic oxidation and reduction peaks associated with electrochemical reactions in PBS. It is clear from

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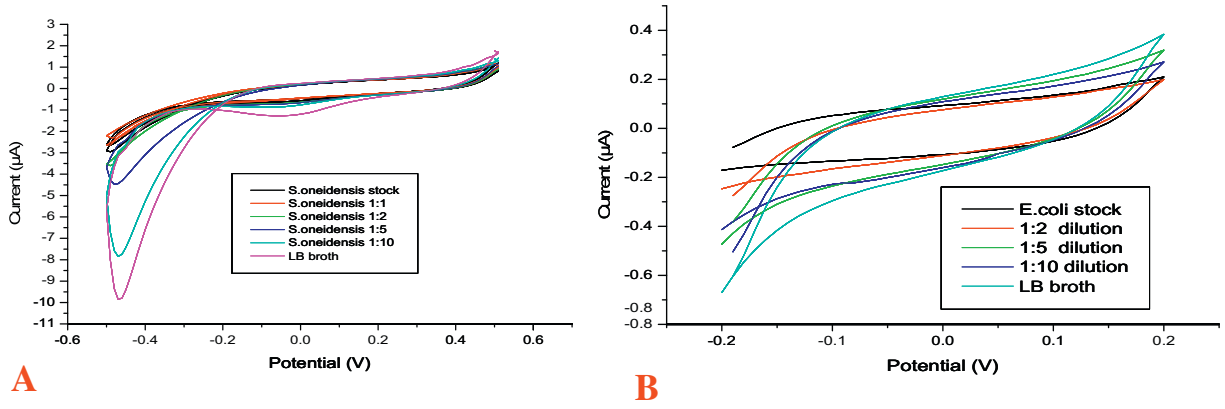

Fig. 4. Cyclic voltammograms recorded on *S. oneidensis* (A) and *E. coli* (B) of different dilutions (1:10, 1:5, 1:2, 1:1, and stock solutions); CV curves for clear LB broth are shown on both graphs.

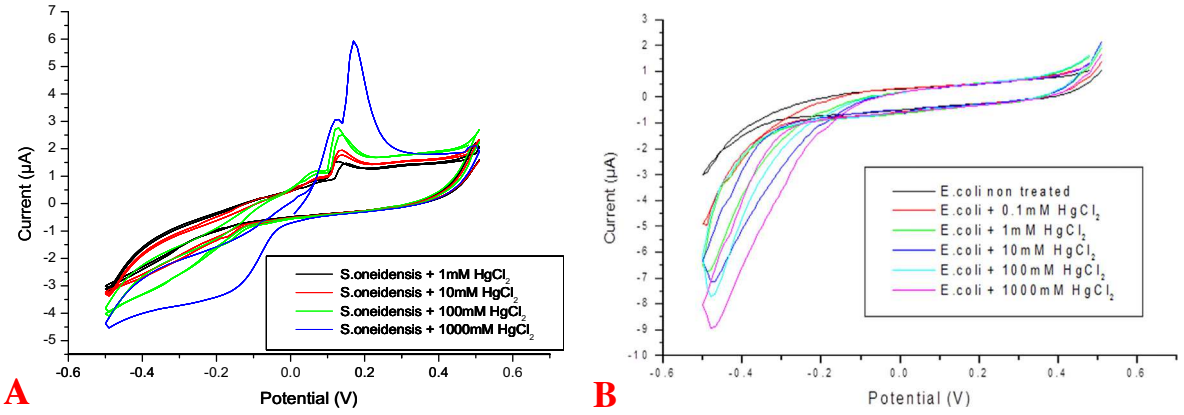

Fig. 5. Cyclic voltammograms of *S. oneidensis* (A) and *E. coli* (B) bacteria solutions in LB broth which treated with different concentration of $HgCl_2$.

Fig. 7, that the values of current, for example cathode current peak at about −0.2 V, decreases with the increase in concentration of immobilized bacteria. Similarly to previous experiments with bacteria solutions, bacteria adsorbed on the surface act as insulator reducing the current.

The next set of data in Fig. 8 was obtained on gold electrodes with both bacteria immobilized from respective stock solutions and then treated with $HgCl_2$ solutions of different concentrations. All measurements were carried out in PBS.

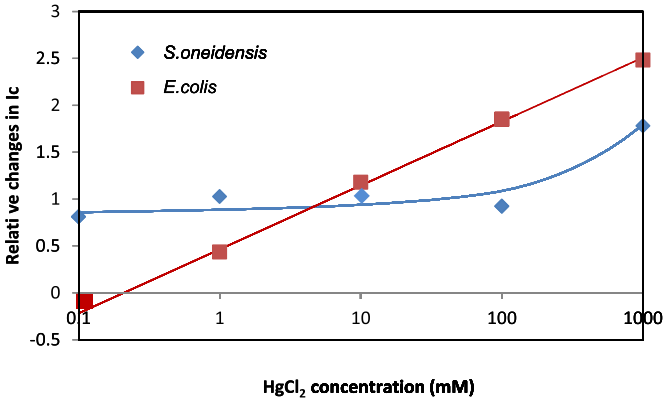

Fig. 6. The dependence of relative changes in cathode current at −0.5 V for both *S. oneidensis* and *E. coli* on the concentration of $HgCl_2$.

For both types of bacteria, the cathode current I_c at −0.5 V increases after treatment with $HgCl_2$ salt (the higher the $HgCl_2$ concentration - the higher I_c). Relative changes of I_c, e.g. for both bacteria vs concentration of $HgCl_2$ are shown in Fig. 9. Here, the values of I_c before the $HgCl_2$ treatment were used as reference.

As one can see, the effect is similar to that observed on liquid bacteria samples. The values of $\Delta I_c/I_c$ for *E. coli* increase steadily with the increase in $HgCl_2$ concentration, while *S. oneidensis* bacteria are practically unaffected by $HgCl_2$ in a wide concentrations range apart from a slight increase in $\Delta I_c/I_c$ at 0.1 mM. High concentration of $HgCl_2$, however, causes the damage to *S. oneidensis* bacteria cell membrane, and they sharply become more conductive.

4. Conclusions and future work

The effect of heavy metals (Hg in this case) on two types of bacteria, e.g. *E. coli* and *S. oneidensis* was studied using three different optical techniques: fluorescent microscopy and flow cytometry which yields directly the ratio of live/dead bacteria, stained, respectively, with "green" and "red" fluorescent dyes as well as optical density measurements at 600 nm. All three optical methods are capable of detecting the effect of heavy metals on the above bacteria, though the flow cytometry is much more reliable. The results obtained were encouraging, however the use of expensive and bulky optical instrumentation is not the way forward for portable and cost-effective sensor development.

Simple electrochemical tests, e.g. cyclic voltammograms, either on gold electrodes immersed into liquid bacteria samples or (even better)

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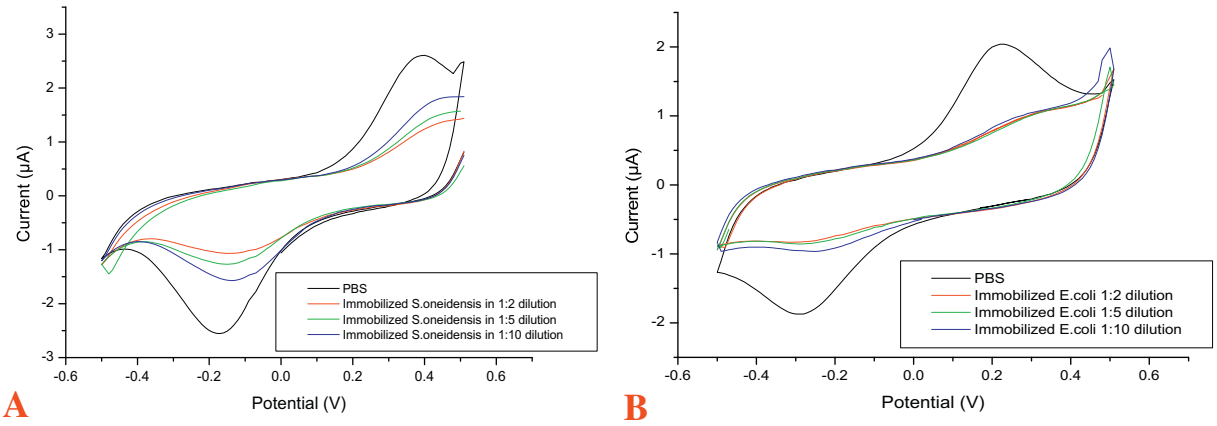

Fig. 7. CVs of *S. oneidensis* (A) and *E. coli* (B) bacteria immobilized from their respective solutions of different concentrations.

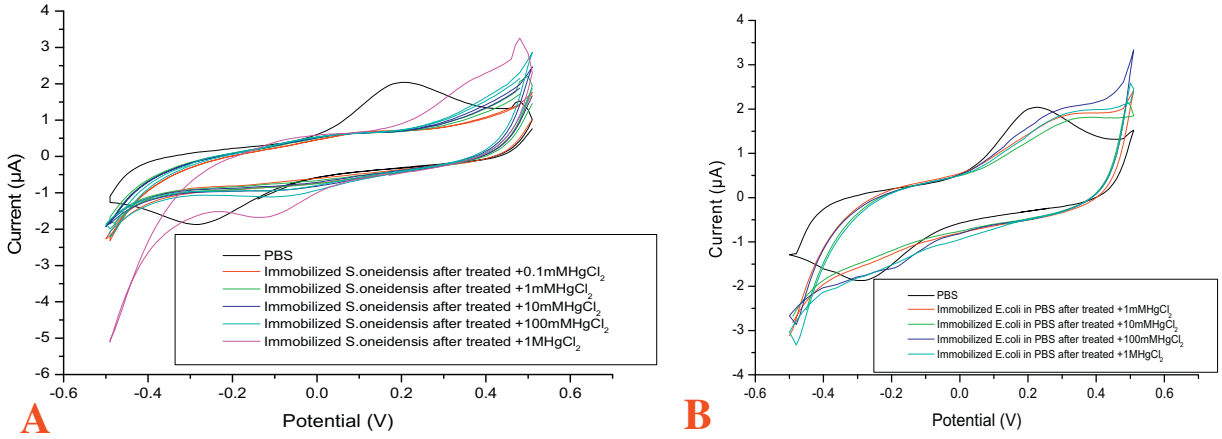

Fig. 8. CVs recorded on gold electrodes with immobilized *S. oneidensis* (A) and *E. coli* (B) bacteria in PBS, both before and after exposure to different concentrations of $HgCl_2$.

on gold electrodes with immobilized bacteria appeared to be very successful. The values of cathode current was found to correlate with bacteria concentration and thus with the concentration of $HgCl_2$ salt acting as inhibitor for bacteria. The effect of $HgCl_2$ on the two bacteria used was different; *E. coli* is strongly inhibited by $HgCl_2$, while *S. oneidensis* is practically unaffected in a wide concentration range of $HgCl_2$. The latter fact opened possibility of exploiting the principles of pattern recognition for identification of pollutants.

This work proved the concept of a novel, simple and cost effective electrochemical bacteria-based sensor and sensor array for preliminary assessment of the presents of pollutants in water. Future work which is currently underway will focus on extending the range of pollutants to pesticides and petrochemicals as well as adding another type of bacteria to the sensor array.

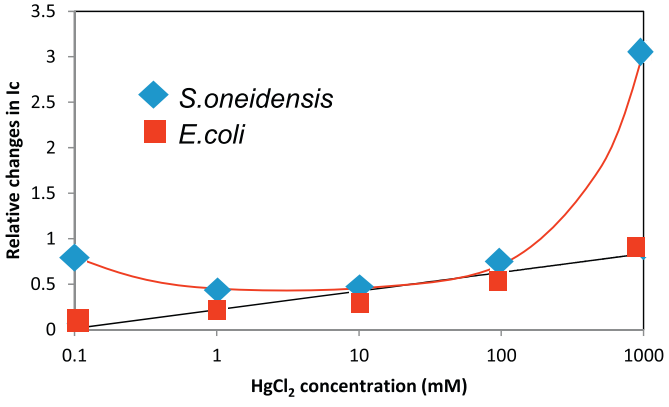

Fig. 9. The dependencies of relative changes of I_c of gold electrodes with immobilized *S. oneidensis* and *E. coli* bacteria vs. concentration of $HgCl_2$.

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